BIOLOGIA PLANTARUM 52 (1): 129-132, 2008

BRIEF COMMUNICATION

Growth and ultrastructural characteristics of *Citrus* cells grown in medium containing NaCl

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Abstract

Changes in growth and structural properties of *Citrus* cell line Carvalhal acclimated to 100 mM NaCl in the medium were compared to unacclimated control cells and cells exposed to 100 mM NaCl. Transmission electron microscopy (TEM) showed presence of ring-shaped mitochondria, increase in the number of amyloplasts and lipid bodies, higher cell wall thickness and partitioned vacuoles in acclimated cells.

Additional key words: acclimation, cell culture, cell wall, mitochondria, salt stress, transmission electron microscopy.

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Citrus is grown preferentially in semi-arid areas where irrigation is required to produce maximum yield. In these areas, irrigation water contains dissolved salt that accumulate in soil and cause salinity related reduction in productivity. Cells in culture are simple systems that offer a suitable alternative to study physiological mechanisms of tolerance to salinity.

The physiological and biochemical aspects of salt tolerance in plants have attracted considerable interest (Gosset *et al.* 1996, Passos *et al.* 2005, Mandhania *et al.* 2006). Plant cells adapted to salinity undergo numerous physiological and morphological changes and are able to grow and multiply in the presence of otherwise lethal concentrations of salt (Binzel *et al.* 1988). The *in vitro* selection of cells tolerant to salinity and regeneration of plants opens new perspectives for study of adaptive mechanisms in metabolism and cellular structure. In plants exposed to salt stress an increase in cell wall thickness, accumulation of rough endoplasmatic reticulum, alteration of number and ring-shape of mitochondria, alteration of composition and structure of cellular membranes and changes in number of lipid bodies have been observed (Bressan *et al.* 1990, Piqueras *et al.* 1994, Mansour and Salama 2004). The objective of this study is to produce salt-acclimated *Citrus* cell suspension culture and investigate growth and ultrastructural properties of the acclimated cells that may have adaptive value for *Citrus* plants.

Cell suspension cultures of *Citrus* cv. Carvalhal tangor were propagated on a rotary shaker (140 rpm), at 24 ºC in the dark, in the Murashige and Skoog (1962; MS) liquid medium containing 1 mg dm^{-3} kinetin, 0.1 mg dm^{-3} nicotinic acid, 0.4 mg dm^{-3} thiamine-HCl, 0.5 g dm^{-3} malt extract and 50 g dm^{-3} sucrose, pH adjusted to 5.7. Cultures were maintained in 500 cm^3 Erlenmeyer flasks and subcultured weekly onto fresh medium (20 % v/v). For salt-acclimation, friable calli of *Citrus* kindly provided by Dr. José Leitão (Universidade do Algarve) were exposed to the MS medium containing 100 mM NaCl for at least 6 months and transferred to fresh medium every 3 weeks. After 6 months of sub-culture in MS agar medium supplemented with 100 mM NaCl, salt-acclimated calli with growth properties similar to unacclimated calli were obtained. The friable salt-acclimated calli were transferred to liquid medium supplemented with 100 mM NaCl and subcultivated weekly until homogenous suspension culture was obtained after 7 passages in medium containing 100 mM NaCl.

Rate of cell growth in suspension cultures of control cell line (non-acclimated cells) was compared to the salt-acclimated cell line and control cells exposed to 100 mM NaCl (salt-shock cells). Fresh mass (FM) of 10 cm^3

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Received 5 June 2006, *accepted* 1 December 2006.
Abbreviation: TEM - transmission electron microscopy.
Acknowledgements: ALF was the recipient of a PhD grant (Praxis XXI/BD/21234/99) from Fundação para a Ciência e a Tecnologia (Portugal). We also want to thank Drs. N.K. Singh and M. Miller (Auburn University) for their assistance in transmission electron microscopy. The authors wish to thank Dr. José Leitão (Universidade do Algarve) for providing the 'Carvalhal' tangor calli.
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culture was determined after filtration of cells on *Whatman* filter paper. Dry mass (DM) was determined after drying fresh cell material at 80 ºC, until constant mass. The results are means of three replicates. Cells were collected by centrifugation at 1 500 *g* and fixed in respective growth media containing 2.5 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.0. Fixed cells were rinsed in chilled cacodylate buffer for 10 min, postfixed in 1 % osmium tetroxide in the same buffer for 4 h at 4 ºC. After postfixation, cells were briefly rinsed in cacodylate buffer, dehydrated in a graded series of ethanol and soaked in propylene oxide for 15 min. Dehydrated cells were embedded in *Epon 825* (*Hexion RP3073-01*). Thin sections of cell samples (six independent samples) were cut with a *Leica Ultracut T* ultramicrotome (Deerfield, USA), transferred to 100-mesh copper grids and stained with uranyl acetate and lead citrate. The sections were examined with a *Zeiss 10-CR* transmission electron microscope (Obercochen, Germany) operated at an accelerating potential of 60 kV. The results were subjected to analysis of variance (*ANOVA*) and mean values were compared using *SPSS for Windows* (statistical program). Duncan Post-hoc tests were performed when significant differences occurred at 5 % level.

Growth characteristics of the non-acclimated, salt-acclimated and salt shocked cultures were comparable but salt-acclimated cultures showed a higher biomass accumulation [59.0 g dm^{-3}(FM)] as compared to non-acclimated [47.1 g dm^{-3}(FM)] and salt-shocked [41.3 g dm^{-3}(FM)] cultures (Table 1). The growth properties of salt acclimated cells suggest that these cells have acquired metabolic capacity to grow in saline environment. Antioxidant enzyme system appears to be involved in the increased tolerance of *Zea mays* L. (Neto *et al.* 2005) and of *Citrus* cell suspensions (Ferreira and Lima-Costa 2006). The reduction of biomass observed is probably due to osmotic stress. A biochemical alterations, *e.g.*, the different polysaccharides composition of the matrix and synthesis of cell wall proteins were also observed (Chang *et al.* 1996, Yang and Yen 2002).

Electron microscopic analyses of *Citrus* unacclimated and acclimated cells show significant cytological differences resulting from salt-acclimation (Fig. 1*A,D,H*). Unacclimated cells showed dense cytoplasm with abundant mitochondria, amyloplasts, lipid bodies, vacuoles, abundance of rough endoplasmic reticulum, and normal nuclei with dense nucleoli (Fig. 1*A,B,C*). In the salt-shocked cells, number of mitochondria was increased and lipid bodies and amyloplasts were seen (Fig. 1*H-J*). The increase in the number of lipid bodies observed in salt-shocked and also in acclimated cells may be related to the alterations of lipids in the plasma membrane by exposure to NaCl. The stored lipids may serve as the reserve for cells under stress. The contact with saline environments affects the structure and chemical composition of the membranes (Kuiper 1984, Kerkeb *et al.* 2001).

Table 1. Effect of NaCl on accumulation of fresh mass and cell wall thickness of *Citrus* suspension cultures. Each value is the mean ± SE of three replicates.

Cell line	NaCl [mM]	FM [g dm^{-3}]	Wall thickness [μm]
Non-acclimated	0	47.1	0.26 ± 0.035
Salt-shocked	100	41.3	0.19 ± 0.018
Salt-acclimated	100	59.0	0.33 ± 0.029

Mitochondria of salt-acclimated and salt-shocked cells show a similar ring-shaped morphology (Fig. 1*I,J*). This may be connected with an adaptive alteration in the protein synthesis or metabolite composition of the cytoplasm. Those alterations seem to have a transitory character used by the cells as a strategy to keep the metabolism under salt stress. Acclimated cells show partitioned vacuoles with an average of two vacuoles per cell (Fig. 1*E*). This vacuolar subdivision is compatible with a more efficient compartmentation of the toxic ions (Binzel *et al.* 1988, Chinnusamy *et al.* 2005). The increase in toxic ion content in the cytoplasm and thus avoiding salt toxicity is prevented mainly by Na^+/H^+ antiport in the tonoplast and also by uptake of water (Hasegawa *et al.* 2000). Acclimated cells had higher number of amyloplasts (Fig. 3*D,E,G*) than unacclimated cells which is compatible with the higher energy required for maintenance of this cell culture. The acclimation of plant cells requests the production and accumulation of osmotically active solutes, such as organic acids, amino acids, or sugars (Yeo 1998, Hasegawa *et al.* 2000), that lead to the osmotic adjustment of the cell, but also the accumulation of the sugar reserve in the form of starch. The accumulation of starch under abiotic stress has been reported previously (Huang and Liu 2002) and it is tempting to speculate that starch synthesis from sucrose play a role in moderating the hyperosmotic condition. In comparison to unacclimated cells, acclimated cells had thicker cell walls of approximately 0.3 μm (Fig. 1*F*, Table 1). An increase in the thickness of the cell wall was shown earlier in salt-adapted tobacco cells (Bressan *et al.* 1990) and in lemon cells (Piqueras *et al.* 1994). This modification can occur as a mechanism to preserve the pressure potential of the cell in NaCl containing medium (Singh *et al.* 1989).

The dynamic process of salt acclimation is multiphasic and the physiological response correlates with changes in the cell ultrastructure. Salt acclimated cells show absence of necrosis and high growth rate, and higher biomass accumulation than salt-shocked cells. These cells also show important ultrastructural modifications, such as, increase in number of amyloplasts and lipid bodies, ring-shaped mitochondria, and increase in the cell wall thickness. These properties may be responsible for their ability to withstand growth in saline environment.

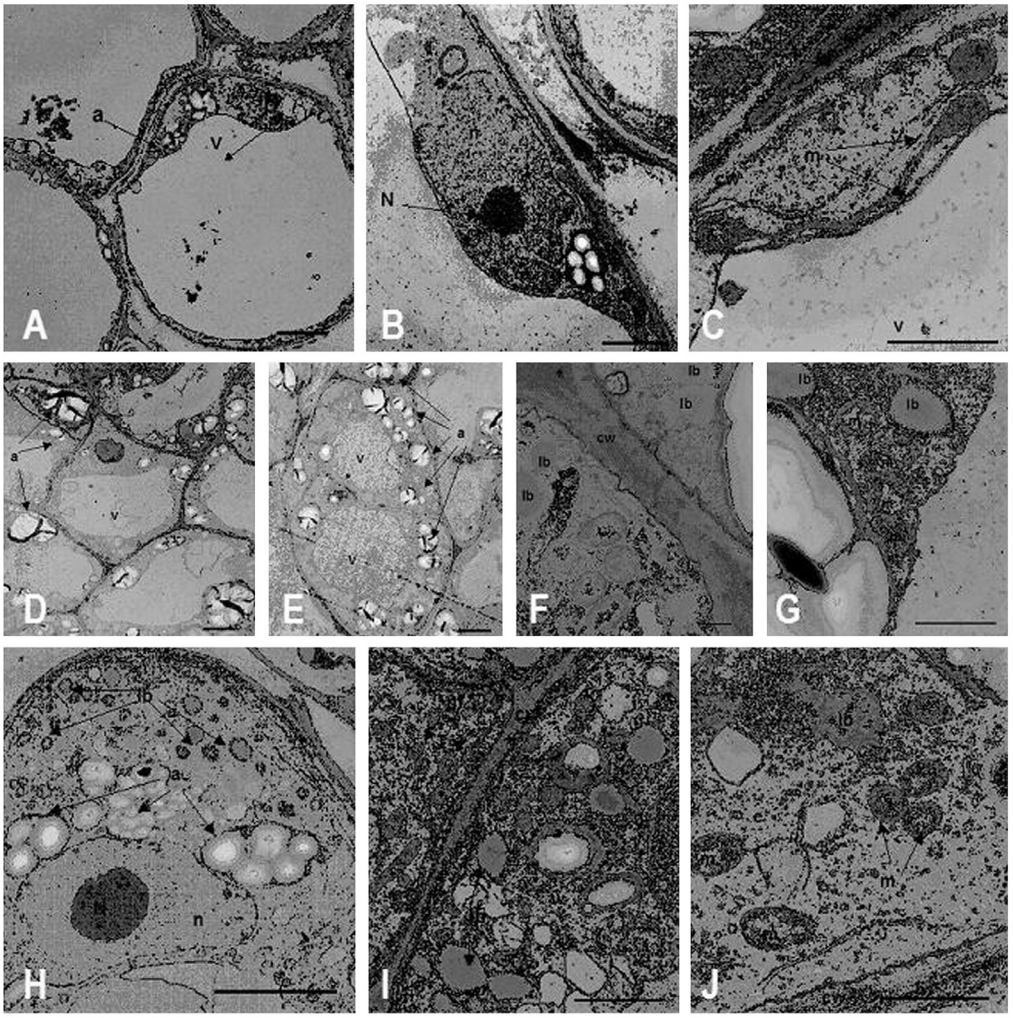

Fig. 1 *A - C.* Ultrastructure of *Citrus* cells unacclimated to NaCl: *A* - general view (*bar* = 5 μm, ×2 000), *B* - a detail of a nucleus (*bar* = 2 μm, ×8 000) and *C* - mitochondria (*bar* = 1 μm, ×25 000). *D - G.* Structure of salt-acclimated cells: *D* - general view (*bar* = 5 μm, ×5000), *E* - two vacuoles per cell and amyloplasts (*bar* = 5 μm, ×5000), *F* - cell wall (*bar* = 5 μm, ×20 000), *G* - lipid bodies and mitochondria (*bar* = 5 μm, ×25 000). H - J. Structure of salt-shocked cells: *H* - general view (*bar* = 5 μm, ×5 000), *I* - cell wall (*bar* = 1 μm, ×20 000) and *J* - mitochondria and lipid bodies (*bar* = 1 μm, ×25 000). N - nucleoli, n - nucleus, a - amyloplasts, lb - lipid bodies, cw - cell wall, m - mitochondria, v - vacuole.

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